

# Transient mitochondrial permeability transition pore opening after neonatal cardioplegic arrest

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**Background:** Neonatal cardioplegic arrest is associated with apoptosis-related mitochondrial dysfunction, including Bax translocation to the mitochondria, mitochondrial permeabilization, cytochrome c release, and electron transport chain dysfunction. We sought to characterize the time course and mode of postcardioplegic mitochondrial membrane permeabilization and hypothesize that permeabilization is transient and mediated by the mitochondrial permeability transition pore.

**Methods:** Isolated, perfused neonatal rabbit hearts underwent 60 minutes of warm crystalloid cardioplegic arrest followed by 120 minutes of reperfusion. Mitochondrial permeabilization was evaluated by means of infusion of 2-deoxy [ $^3\text{H}$ ] glucose and subsequent detection of entrapment in isolated mitochondrial fractions. Groups included preloading with 2-deoxy [ $^3\text{H}$ ] glucose followed by cardioplegia and reperfusion (CCP), cardioplegia and cyclosporin A (specific inhibitor of mitochondrial permeability transition pore opening; CCP + CsA) or HA14-1 (Bcl-2 inhibitor; CCP + HA), and noncardioplegia control hearts (non-CCP). Reconstitution of mitochondrial integrity was tested by means of delayed infusion of 2-deoxy [ $^3\text{H}$ ] glucose 30 minutes after reperfusion (P-CCP).

**Results:** Cardioplegic arrest was associated with mitochondrial permeability transition pore opening, Bax translocation, cytochrome c release, radical oxygen species production, and electron transport chain dysfunction. Inhibition of mitochondrial permeability transition pore opening by cyclosporin A ameliorated this response, whereas inhibition of Bcl-2 exacerbated these changes. Postreperfusion entrapment of 2-deoxy [ $^3\text{H}$ ] glucose was significantly reduced in comparison with that seen in CCP hearts, suggesting that closure of the mitochondrial permeability transition pore ensues within 30 minutes after reperfusion.

**Conclusions:** Apoptosis-related mitochondrial dysfunction in postcardioplegic neonatal hearts is mediated by mitochondrial permeability transition pore opening, which is transient and associated with deficits in electron transport. Clinical strategies directed to minimize mitochondrial permeability transition pore opening are likely to improve postoperative myocardial dysfunction after neonatal cardiac surgery. (*J Thorac Cardiovasc Surg* 2011;141:975-82)

As a critical mediator in apoptosis signaling pathways, mitochondria integrate upstream death stimuli and undergo structural and functional remodeling, with subsequent transmission of apoptotic signals to downstream executioner proteins.<sup>1</sup> After protected ischemia (cardioplegic arrest) and reperfusion, we previously reported a constellation of apoptosis-related alterations, including Bax translocation, mitochondrial outer membrane permeabilization, cytochrome c release, and deficits in electron transport in the postoperative myocardium.<sup>2</sup> These alterations, collectively termed apoptosis-related mitochondrial dysfunction,

are detectable within hours of cardioplegic arrest and are associated with deficits in global myocardial performance in laboratory models<sup>2,3</sup> and might contribute to the postoperative myocardial dysfunction observed in neonatal patients after cardiac surgery.<sup>4</sup> Because of the close interplay between mitochondrial structure and energy production,<sup>5</sup> preservation of mitochondrial architectural and functional integrity is an important cardioprotective strategy in the postoperative neonatal heart.<sup>3</sup>

The mitochondrial permeability transition pore (MPTP) is a nonspecific pore spanning the inner and outer mitochondrial membrane, and its opening during reperfusion results in impairment of mitochondrial integrity and, under some circumstances, cell death.<sup>6</sup> Depending on the extent of ischemia-reperfusion injury, MPTP opening is subclassified into transient opening or irreversible, long-lasting opening, and the duration of MPTP opening is a mediator of cell fate (apoptosis vs necrosis),<sup>7</sup> which corresponds to cardiac performance (reversible vs irreversible cardiac functional recovery). We have previously demonstrated that selective inhibition of MPTP opening after cardioplegic arrest leads

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# Abbreviations and Acronyms

CS	= citrate synthase
CsA	= cyclosporin A
EGTA	= ethyleneglycol-bis-( $\beta$ -aminoethylether)-N,N,N',N'-tetraacetic acid
KHB	= Krebs–Henseleit buffer
MOPS	= 3-morpholinopropane-1-sulfonic acid
MPTP	= mitochondrial permeability transition pore
OAA	= oxaloacetic acid
ROS	= reactive oxygen species
SSM	= subsarcolemmal mitochondria
[ <sup>3</sup> H] DOG	= 2-deoxy [ <sup>3</sup> H] glucose

to improved preservation of myocardial performance after reperfusion.<sup>3</sup>

Bax typically resides in the cytoplasm and is a strongly proapoptotic member of the Bcl-2 family. Bax activity is opposed by Bcl-2, an antiapoptotic member of the Bcl-2 family. Overexpression of Bax is associated with an exaggerated response to ischemia–reperfusion injury.<sup>8</sup> After an apoptotic stimulus, Bax translocates from the cytoplasm to the mitochondria and participates in MPTP-mediated mitochondrial permeabilization.<sup>9</sup> MPTP opening can be initiated with non-specific stimuli associated with reperfusion (eg, oxidative stress or high calcium concentrations), and the permeabilization stimulus can then be further enhanced through participation of Bax.<sup>6</sup> In contrast, other investigators have demonstrated that Bax oligomers have pore-forming capacity, which is independent of MPTP inhibition with cyclosporin A (CsA).<sup>10</sup> Thus the roles of the MPTP and Bax in postreperfusion mitochondrial permeabilization are incompletely defined but important mediators of mitochondrial permeabilization after ischemia–reperfusion injury.

Because mitochondrial permeabilization is associated with diminished postoperative global myocardial performance, which typically recovers in the early postoperative period,<sup>4</sup> we hypothesized that the MPTP opening is a critical and transient event in apoptosis-related mitochondrial dysfunction. We tested this hypothesis in a neonatal cardiac model of cardioplegic arrest using 2 agents that target the MPTP: CsA, which is known to ameliorate reperfusion injury in vitro and in vivo in animal models by means of specific inhibition of MPTP opening,<sup>11,12</sup> and HA14-1, a promoter of mitochondrial permeabilization that selectively inhibits Bcl-2, an antiapoptotic protein associated with inhibition of MPTP opening.<sup>13</sup> The interaction of these agents with the MPTP was evaluated by using the radioactive probe 2-deoxy [<sup>3</sup>H] glucose ([<sup>3</sup>H] DOG), which enters and is trapped in the

mitochondria when the MPTP undergoes reversible opening, with the overall objective to gain insights into the time course of MPTP opening after cardioplegic arrest.

## MATERIALS AND METHODS

### Experimental Model

New Zealand white neonatal rabbits (age, 6 days; weight, 150–200 g) were anesthetized with sodium pentobarbital (25 mg/kg administered intraperitoneally), anticoagulated with heparin (1000 U/kg administered intraperitoneally), and ventilated. The aorta was cannulated and the heart was retrogradely perfused in situ to avoid ischemia. The heart (net weight, approximately 1 g) was then excised, mounted on a Langendorff apparatus, and perfused at 75 mm Hg perfusion pressure with Krebs–Henseleit buffer (KHB; 118 mmol/L NaCl, 2.1 mmol/L NaHCO<sub>3</sub>, 1.2 mmol/L KH<sub>2</sub>O<sub>4</sub>, 4.7 mmol/L KCl, 1.2 mmol/L MgSO<sub>4</sub>, 1.8 mmol/L CaCl<sub>2</sub>, and 11 mmol/L glucose). Perfusate was filtered with a 4- $\mu$ m filter, equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 37°C, and adjusted to a pH of 7.35 to 7.4.

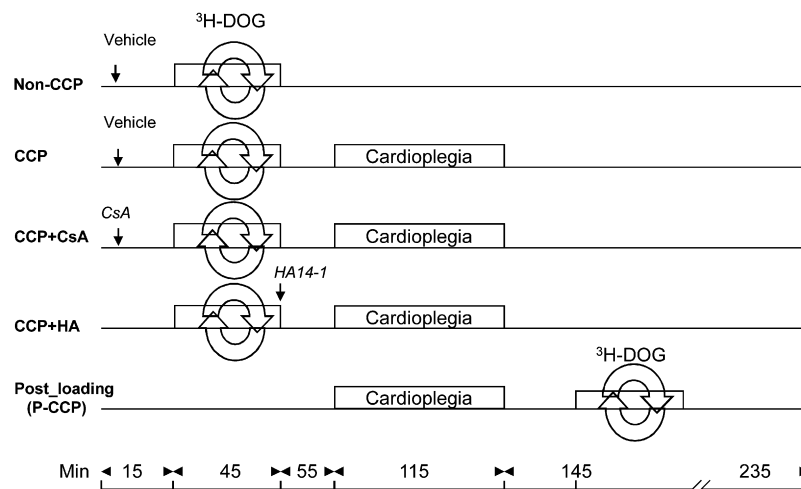
All animals received humane care in compliance with the “Guide for the care and use of laboratory animals” prepared by the Association for Assessment and Accreditation of Laboratory Animal Care (March 1999).

### Experimental Protocol

After a 15-minute flow-through period for stabilization, hearts were perfused in recirculation mode with 200 mL of KHB containing 1 mmol/L [<sup>3</sup>H] DOG (0.2  $\mu$ Ci<sup>-1</sup>) for 30 minutes. Perfusion was then switched back to flow-through mode with normal KHB for 10 minutes, followed by crystalloid cardioplegic arrest for 1 hour (70 mL/kg Plegisol; Hospira, Inc, Lake Forest, Ill) at 37°C and 120 minutes of reperfusion (Figure 1). For CsA-treated hearts (CCP+CsA group, n = 6), CsA (0.2  $\mu$ mol/L) was added to the KHB during the stabilization period, as previously described.<sup>3</sup> For HA14-1-treated hearts (CCP + HA group, n = 6), HA14-1 (20  $\mu$ mol/L) was administered 10 minutes before cardioplegic arrest, as previously described.<sup>13</sup> Control hearts included hearts undergoing cardioplegic arrest and hearts that were perfused but did not undergo cardioplegic arrest. Cardioplegic control hearts were perfused with KHB and vehicle (0.01% dimethyl sulfoxide) without either drug and underwent identical periods of cardioplegic arrest and reperfusion (CCP group, n = 6). Noncardioplegic control hearts (non-CCP group, n = 6) were preloaded with [<sup>3</sup>H] DOG without cardioplegic arrest and underwent uninterrupted perfusion. To assess late opening of the MPTP, another set of control hearts underwent cardioplegic arrest using the same protocol with the exception that they were loaded with [<sup>3</sup>H] DOG 30 minutes after reperfusion in recirculation mode followed by another 60 minutes of flow-through perfusion with KHB (postloading control [P-CCP] group, n = 6). At the end of all experiments, ventricular tissue was weighed and immediately fractionated for subsarcolemmal mitochondrial (SSM) assessment followed by mitochondrial functional assessment and MPTP opening measurement.

### Mitochondrial Isolation

Whole ventricular tissue was used for SSM isolation, as described by Palmer and colleagues.<sup>14</sup> SSM were specifically examined because previous reports have demonstrated that SSM are more sensitive to ischemia–reperfusion injury than interfibrillar mitochondria and are postulated to be more sensitive mediators of the mitochondrial interaction with reperfusion injury mechanisms within the myocyte.<sup>15</sup> The ventricular tissue was weighed, minced in buffer A (100 mmol/L KCl, 50 mmol/L 3-morpholinopropane-1-sulfonic acid (MOPS), 1.0 mmol/L MgSO<sub>4</sub>, 1.0 mmol/L ethyleneglycol-bis-( $\beta$ -aminoethylether)-N,N,N',N'-tetraacetic acid [EGTA], and 1 mmol/L adenosine triphosphate [pH 7.4]) at 4°C, and then homogenized in buffer A with 0.2% bovine serum albumin followed by 2 loose strokes with a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 500g, and the supernatant was saved and combined with the



**FIGURE 1.** Experimental design showing time course of perfusion of isolated hearts in the noncardioplegia control (*non-CCP*), preloading with 2-deoxy [ $^3\text{H}$ ] glucose followed by cardioplegia and reperfusion (*CCP*), cardioplegia and cyclosporin A (*CCP+CsA*), cardioplegia and HA14-1 (*CCP+HA*), and delayed infusion of 2-deoxy [ $^3\text{H}$ ] glucose 30 minutes after reperfusion (*P-CCP*) groups.  $^3\text{H}$ -DOG, 2-Deoxy ( $^3\text{H}$ ) glucose.

supernatant from the second spin after the pellet was resuspended. The pooled supernatant was centrifuged at 3000g to sediment SSM, which was washed twice and resuspended in KME buffer (100 mmol/L KCl, 50 mmol/L MOPS, and 0.5 mmol/L EGTA).

### Mitochondrial Permeabilization and Cytochrome c Content

The integrity of the mitochondrial outer membrane was assessed by measuring the boost of oxygen consumption of mitochondrial complex IV after administration of exogenous cytochrome c into the respiratory chamber. The subsequent increase in complex IV activity reflects the permeabilization of the outer mitochondrial membrane.<sup>16</sup>

Mitochondrial cytochrome c content was determined by using the Agilent 8453 UV-visible Spectrophotometer (Agilent Technologies, Waldbronn, Germany) with dual-cuvette methods, as described by William.<sup>17</sup> Each cuvette contained 0.5 mg of mitochondria solubilized in 5% deoxycholate in 10 mmol/L sodium phosphate (pH 7.0). The oxidized cuvette contained 5 mmol/L potassium ferricyanide, and the reduced cuvette contained 5 mmol/L ascorbate, to which several grains of sodium dithionite were added. The cytochrome c content was determined by the difference of oxidized and reduced spectra and standardized by means of mitochondrial citrate synthase (CS) activity.

### Mitochondrial Oxygen Consumption Measurement

Isolated mitochondrial oxygen consumption was measured in buffer containing 100 mmol/L KCl, 50 mmol/L MOPS, 1.0 mmol/L EGTA, 5.0 mmol/L  $\text{KH}_2\text{PO}_4$ , and 5% defatted bovine serum albumin at 30°C by using a Clark-type electrode (Instech Laboratories, Inc, Plymouth, Pa). As shown in Figure 2, mitochondrial respiratory state 2 and state 3 for complexes I, II, and IV were measured in the presence of sequential administration of substrates and inhibitors (glutamate and malate for complex I, rotenone and succinate for complex II, and antimycin, N,N,N',N'-tetramethyl-*p*-phenylenediamine, and ascorbate for complex IV) added in the following order and final concentrations: 2.5 mmol/L glutamate and 2.5 mmol/L malate, 1 mmol/L lower dose of adenosine diphosphate, 2 mmol/L higher dose of adenosine diphosphate, 2  $\mu\text{mol/L}$  rotenone, 5 mmol/L succinate, 1  $\mu\text{mol/L}$  antimycin A, 1 mmol/L ascorbate, and 0.4 mmol/L N,N,N',N'-tetramethyl-*p*-phenylenediamine. Mitochondrial respiratory activity was expressed as the amount of oxygen (nanomoles)

consumed per minute per milligram of mitochondrial protein. Mitochondrial complexes I, II, and IV respiratory control ratios were expressed as follows: state 3/state 2. The voltage signal was amplified and digitized by using PowerLab/4sp (AD Instruments, Inc, Colorado Springs, Colo). All the substrates and inhibitors were purchased from Sigma-Aldrich (St Louis, Mo).

### In Situ Measurement of MPTP Opening

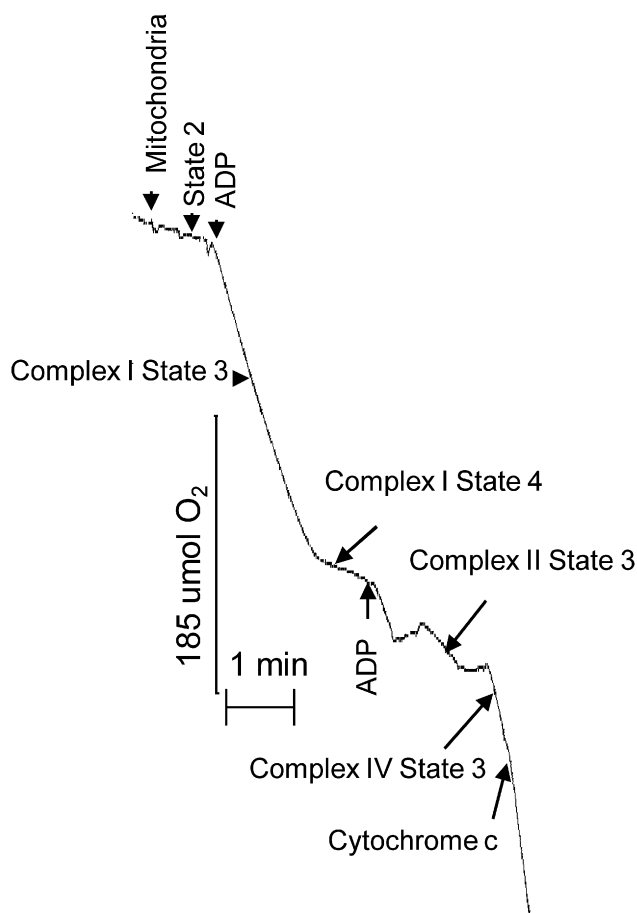
As described previously,<sup>18</sup> hearts were homogenized, and samples of crude ventricular homogenate and mitochondria were assayed for tritium ( $^3\text{H}$ ). The CS activity of mitochondrial fraction was measured. Mitochondrial entrapment of [ $^3\text{H}$ ] DOG 6-phosphate was expressed as  $10^5 \times (\text{Mitochondrial } [^3\text{H}] \text{ dpm per unit of CS})/(\text{Total heart } [^3\text{H}] \text{ dpm [g wet weight]})$ . In this calculation CS activity is used to correct for variations in the recovery of intact mitochondria, and the total homogenate [ $^3\text{H}$ ] takes into account differences in loading of hearts with [ $^3\text{H}$ ] DOG 6-phosphate between experiments.

### CS Activity Assay

SSM (100  $\mu\text{g}$ ) was dissolved in 5% cholate and diluted to 100  $\mu\text{L}$  with KME buffer to make the SSM mixture. The CS activity measurement was performed in a 1-mL reaction buffer containing 1  $\mu\text{g}$  of SSM mixture, 0.1 mmol/L 5,5'-dithio-bis (2-nitrobenzoic acid), 0.4 mmol/L acetyl coenzyme A, and 0.5 mmol/L oxaloacetic acid (OAA) at 37°C. The increase of absorbance at 412 nm caused by the CS activity was recorded with an Agilent 8453 UV-visible Spectrophotometer (Agilent Technologies) every 5 seconds for 2 minutes before and after addition of OAA. The absorbance rate change per minute ( $\Delta A_{412} \text{ nm/min}$ ) was calculated by the subtraction of the  $\Delta A_{412} \text{ nm/min}$  after adding OAA from the  $\Delta A_{412} \text{ nm/min}$  before adding OAA. One unit of CS activity was calculated by using the ratio of  $\Delta A_{412} \text{ nm min}^{-1}$  to extinction coefficient (13.6).

### Detection of Mitochondrial Reactive Oxygen Species Production

The method for reactive oxygen species (ROS) measurement was described by Korge and associates.<sup>19</sup> Briefly, malate/glutamate (2.5 mmol/L each)–energized SSM was incubated in mitochondrial respiratory buffer with 10  $\mu\text{mol/L}$  CM- $\text{H}_2\text{DCF}$  diacetate (Molecular Probes, Eugene, Ore) at room temperature for 30 minutes. ROS production was monitored from



**FIGURE 2.** Representative trace of mitochondrial oxygen consumption in complexes I, II, and IV and before and after administration of exogenous cytochrome c. ADP, Adenosine diphosphate.

reduced dichlorofluorescein oxidation (excitation/emission, 490/525) with a microplate spectrofluorometer (Spectra MAX Gemini EM; Molecular Devices, Sunnyvale, Calif).

### Mitochondrial Bax and Bcl-2 Content

Mitochondrial Bax and Bcl-2 concentrations were examined by means of Western blotting of mitochondrial protein fractions. Briefly, proteins were electrophoresed in 10% sodium dodecylsulfate–polyacrylamide gels, transblotted to nitrocellulose membrane, and probed with anti-rabbit Bax (N-20, 1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, Calif) and anti-rabbit Bcl-2 (N-19, 1:1000 dilution, Santa Cruz Biotechnology). For protein-loading controls, the membranes were stripped and reprobed with anti-mouse cytochrome c oxidase subunit IV (1:10,000 dilution, Molecular Probes). Secondary antibodies were coupled to horseradish peroxidase (anti-mouse IgG, 1:5000 dilution, and anti-rabbit IgG, 1:1000 dilution, Santa Cruz Biotechnology). The resulting autoradiographs were scanned and quantified. The amounts of mitochondrial Bax and Bcl-2 were normalized by cytochrome c oxidase subunit IV to control for variation in mitochondrial protein loading.

### Statistics

Data are expressed as means  $\pm$  standard deviations. Planned comparisons between 2 groups were made with *t* tests, as specified. Multiple-group comparisons were made with analysis of variance with Tukey's test for post hoc comparisons, unless otherwise specified.

## RESULTS

### Mitochondrial Permeabilization and Cytochrome c Retention

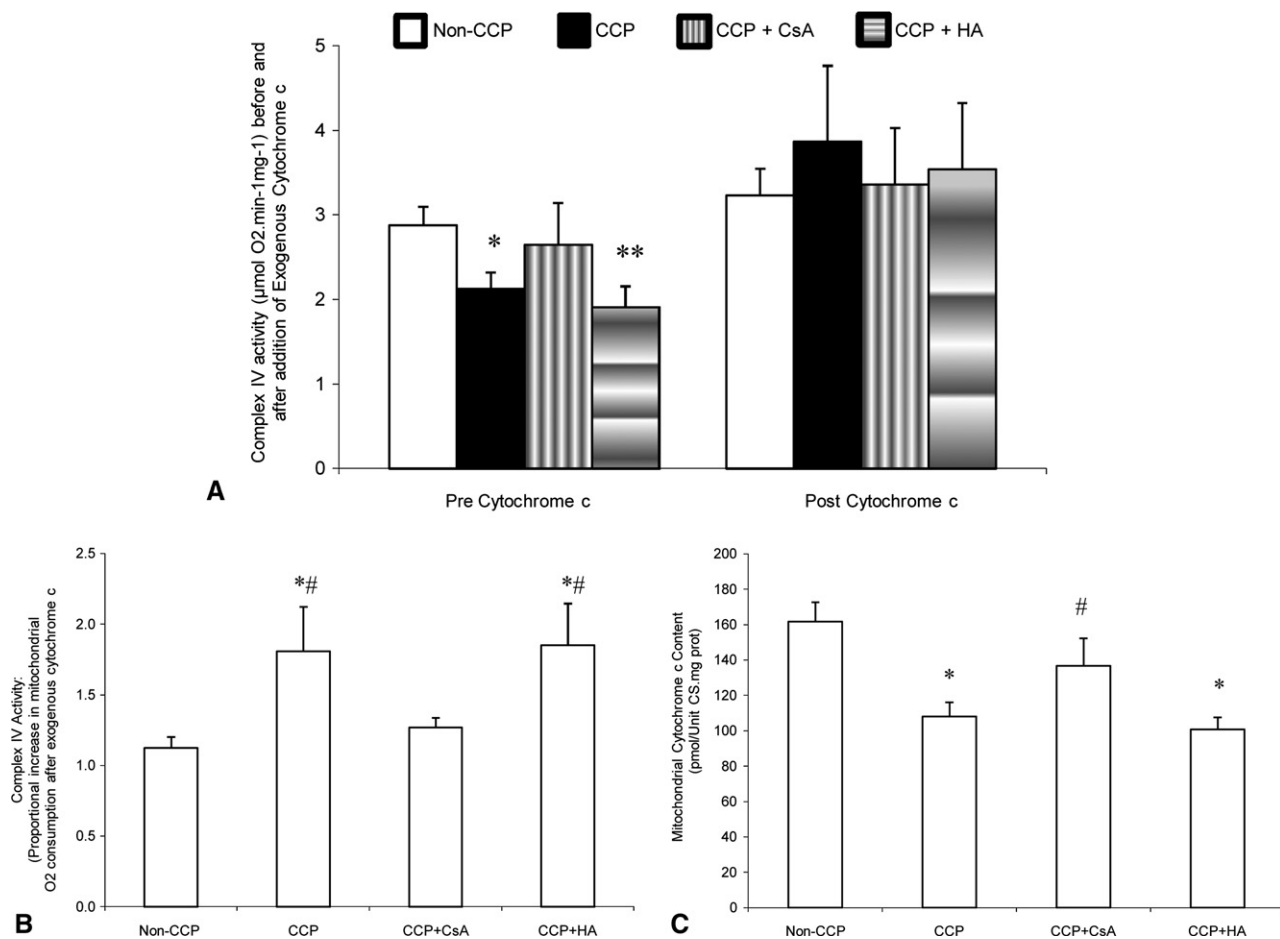
Cardioplegic arrest and reperfusion were associated with a deficit of complex IV activity in CCP and CCP+HA hearts when compared with that seen in non-CCP hearts ( $2.13 \pm 0.19$  and  $1.91 \pm 0.25$  vs  $2.88 \pm 0.22 \mu\text{mol O}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ;  $P < .02$  and  $P < .01$ , respectively), and the complex IV deficit was minimized in CsA-treated hearts (Figure 3, A). After administration of exogenous cytochrome c, the deficit of complex IV activity in CCP and CCP+HA hearts was reversed, and no significant differences in complex IV activity were found among the non-CCP, CCP, CCP+CsA, and CCP+HA groups, indicating that cardioplegic arrest is associated with mitochondrial permeabilization and cytochrome c–dependent reversible deficits in complex IV activity ( $P > .79$  for all comparisons).

Complex IV activity with and without administration of exogenous cytochrome c can be compared as a ratio and used to assess the extent of mitochondrial permeabilization. If the ratio is 1, then exogenous cytochrome c has no effect, and there is no evidence of outer mitochondrial membrane permeabilization. Ratios greater than 1 suggest that exogenous cytochrome c boosts electron transport, and the magnitude of the boost correlates with outer mitochondrial membrane permeabilization.<sup>16</sup> The cytochrome c boost was greater in CCP and CCP + HA hearts than in non-CCP and CCP + CsA hearts ( $1.81 \pm 0.317$  and  $1.85 \pm 0.297$  vs  $1.123 \pm 0.078$  and  $1.269 \pm 0.071$ , respectively;  $P < .01$  all comparisons). There was no significant difference between CsA and Non-CCP mitochondria ( $P = .68$ ; Figure 3, B).

After cardioplegic arrest, cytochrome c was released from the intermembrane space into the cytosol, and the mitochondrial content of cytochrome c was reduced. In comparison with non-CCP mitochondria, cytochrome c content in CCP and CCP + HA mitochondria was lower ( $108.2 \pm 8.1$  and  $100.8 \pm 7.0$  vs  $161 \pm 11.1 \text{ pmol} \cdot \text{CS unit}^{-1} \cdot \text{mg}^{-1} \text{ protein}$ ;  $P < .01$  respectively; Figure 3, C), and CsA-treated mitochondria had better preservation of cytochrome c than CCP mitochondria ( $136.7 \pm 15.7$  vs  $108.2 \pm 8.1 \text{ pmol} \cdot \text{CS unit}^{-1} \cdot \text{mg}^{-1} \text{ protein}$ ,  $P < .01$ ).

### In Situ Measurement of MPTP Opening

Cardioplegic arrest and reperfusion resulted in increased MPTP opening, which was evident in the higher entrapment of [ $^3\text{H}$ ] DOG in CCP hearts compared with that seen in non-CCP hearts ( $67.12 \pm 11.59$  vs  $22.88 \pm 8.63 \text{ dpm} \cdot \text{CS unit}^{-1} \cdot \text{g}^{-1} \text{ wet weight}$ ,  $P < .03$ , Figure 4). Mitochondrial [ $^3\text{H}$ ] DOG entrapment was diminished in CCP+CsA hearts compared with that seen in CCP hearts ( $36.77 \pm 7.71$  vs  $67.12 \pm 11.59 \text{ dpm} \cdot \text{CS unit}^{-1} \cdot \text{g}^{-1} \text{ wet weight}$ ,  $P = 0.03$  by using Fisher's least significant difference test and  $P = .18$  by



**FIGURE 3.** A, Mitochondrial complex IV activity before the addition of exogenous cytochrome c to isolated mitochondria. \* $P < .02$  and \*\* $P < .01$  versus the non-CCP group. B, Fold increase of complex IV activity after addition of exogenous cytochrome c. \* $P < .01$  versus the non-CCP group and # $P < .01$  versus the CCP + CsA group. C, Mitochondrial cytochrome c retention. \* $P < .01$  versus the non-CCP group and # $P < .01$  versus the CCP group. For definitions of the study groups, see the legend for Figure 1.

using Tukey's test) and enhanced in HA14-1-treated hearts compared with CCP hearts ( $105.12 \pm 47.83$  vs  $67.12 \pm 11.59$  dpm · CS unit<sup>-1</sup> · g<sup>-1</sup> wet weight,  $P = .06$ ).

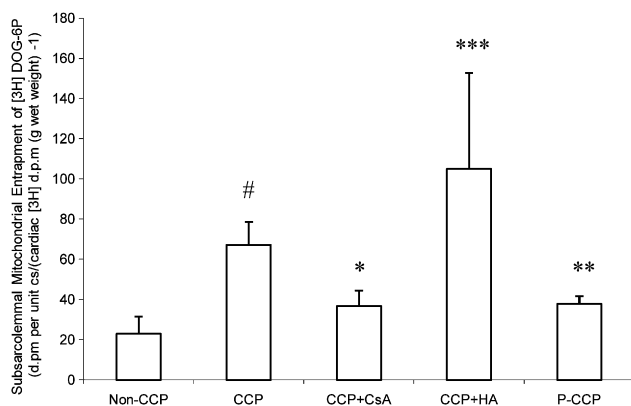
To test whether the MPTP opening in CCP hearts is transient or long lasting, [<sup>3</sup>H] DOG was loaded after hearts had been reperfused for 30 minutes, as previously described by Javadov and coworkers.<sup>18</sup> [<sup>3</sup>H] DOG entrapment in the P-CCP hearts was significantly reduced compared with [<sup>3</sup>H] DOG entrapment in the CCP hearts ( $36.77 \pm 7.71$  vs  $67.12 \pm 11.59$  dpm · CS unit<sup>-1</sup> · g<sup>-1</sup> wet weight,  $P < .05$ ). The [<sup>3</sup>H] DOG entrapment data indicated that cardioplegic arrest and reperfusion induced MPTP opening, which was a transient event, and restoration of mitochondrial integrity was nearly complete after 30 minutes of reperfusion.

### Mitochondrial Oxygen Consumption

The deficit in state 3 respiration for complex I was greater in the mitochondria of CCP and CCP+HA hearts compared

with that seen in non-CCP hearts ( $1439 \pm 446$  and  $851 \pm 381$  vs  $1978 \pm 279$  nmol O<sub>2</sub> · min<sup>-1</sup> · mg<sup>-1</sup> protein;  $P = .059$  and  $P < .01$ , respectively; Table 1). The deficit in the state 3 respiration for complex IV was also present in the mitochondria of CCP and CCP+HA hearts compared with that seen in non-CCP hearts ( $2127 \pm 626$  and  $1907 \pm 390$  vs  $2876 \pm 222$  nmol O<sub>2</sub> · min<sup>-1</sup> · mg<sup>-1</sup> protein;  $P < .02$  and  $.01$ , respectively). Deficiency of state 3 respiration for complex II was only identified in CCP + HA hearts ( $456 \pm 166$  nmol O<sub>2</sub> · min<sup>-1</sup> · mg<sup>-1</sup> protein in CCP + HA hearts vs  $648 \pm 65$  nmol O<sub>2</sub> · min<sup>-1</sup> · mg<sup>-1</sup> protein in non-CCP hearts,  $P < .05$ ). These deficits were minimized in the CCP + CsA hearts, although the protective effect was greatest with complexes I and IV. Cardioplegic arrest and reperfusion decreased the complex I respiratory control ratio in the mitochondria of CCP hearts compared with that seen in non-CCP hearts ( $6.13 \pm 2.45$  vs  $12.36 \pm 2.07$ ,  $P < .01$ , Table 2), whereas the decrease in CCP hearts was significantly reversed in CCP + CsA hearts ( $16.34 \pm 9.93$





**FIGURE 4.** Subsarcolemmal mitochondrial entrapment of 2-deoxy ( $^3\text{H}$ ) glucose ( $^3\text{H}$  DOG). # $P < .03$  versus the non-CCP group and  $*P = .03$ ,  $**P < .05$ , and  $***P = 0.06$  versus the CCP group. For definitions of the study groups, see the legend for Figure 1.

vs  $6.13 \pm 2.45$ ,  $P < .05$ ). The complex IV respiratory control ratio was also decreased in CCP hearts compared with that seen in non-CCP hearts ( $9.19 \pm 2.22$  vs  $18.34 \pm 4.21$ ,  $P < .05$ ), whereas the decrease was significantly reversed in CCP+CsA hearts compared with CCP hearts ( $24.54 \pm 16.19$  vs  $9.19 \pm 2.22$ ,  $P < .05$ ).

### Mitochondrial ROS Production

Cardioplegic arrest and reperfusion resulted in higher mitochondrial ROS production rates in CCP and CCP+HA hearts compared with that seen in non-CCP hearts ( $48.2 \pm 21.4$  and  $45.6 \pm 16.5$  vs  $20.6 \pm 2.4$  RFU/mg protein;  $P < .05$  and  $P = .06$ , respectively), and ROS production was not different when comparing CCP+CsA and non-CCP hearts ( $23.8 \pm 1.6$  vs  $20.6 \pm 2.4$  RFU/mg protein;  $P = .98$ ; Figure 5).

### Mitochondrial Bax and Bcl-2 Content

Mitochondrial Bax concentrations were increased in the mitochondrial fraction in CCP hearts compared with those seen in non-CCP hearts ( $1.06 \pm 0.33$  vs  $0.42 \pm 0.22$  AU,  $P < .05$ ), and CCP+CsA hearts were not statistically different than non-CCP hearts ( $P = .98$ ). There was no difference in Bcl-2 content among these groups (Figure 6).

**TABLE 1.** Mitochondrial oxygen consumption (in nanomoles of oxygen per minute per milligram of protein)

Groups	State 2	State 3		
		Complex I	Complex II	Complex IV
Non-CCP	166 ± 49	1978 ± 279	648 ± 65	2876 ± 222
CCP	240 ± 42	1439 ± 446§	588 ± 212	2127 ± 626†
CCP+CsA	156 ± 99	1816 ± 228	571 ± 132	2650 ± 532
CCP+HA	209 ± 23	851 ± 381*	456 ± 166‡	1907 ± 390*

Values are presented as means ± standard deviations (n = 6 for each group). For definitions of the study groups, see the legend for Figure 1. \* $P < .01$ , † $P < .02$ , ‡ $P < .05$ , and § $P = .059$  versus the non-CCP group.

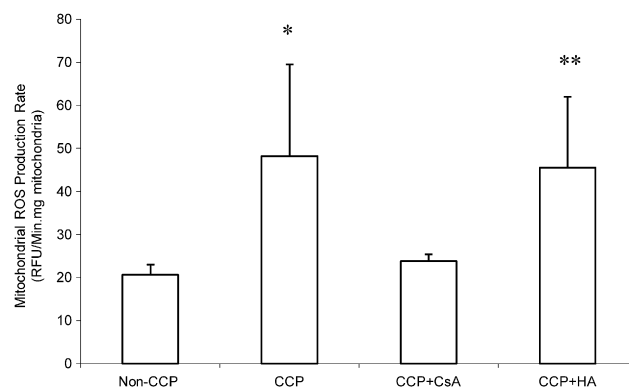
**TABLE 2.** Mitochondrial respiratory control ratio (state 3/state 2)

Groups	Complex I	Complex II	Complex IV
Non-CCP	12.36 ± 2.07	4.08 ± 0.74	18.34 ± 4.21
CCP	6.13 ± 2.45*	2.50 ± 0.45	9.19 ± 2.22†
CCP+CsA	16.33 ± 9.93‡	5.15 ± 3.25§	24.54 ± 16.19‡
CCP+HA	4.09 ± 1.93	2.17 ± 0.74‡	9.24 ± 1.70

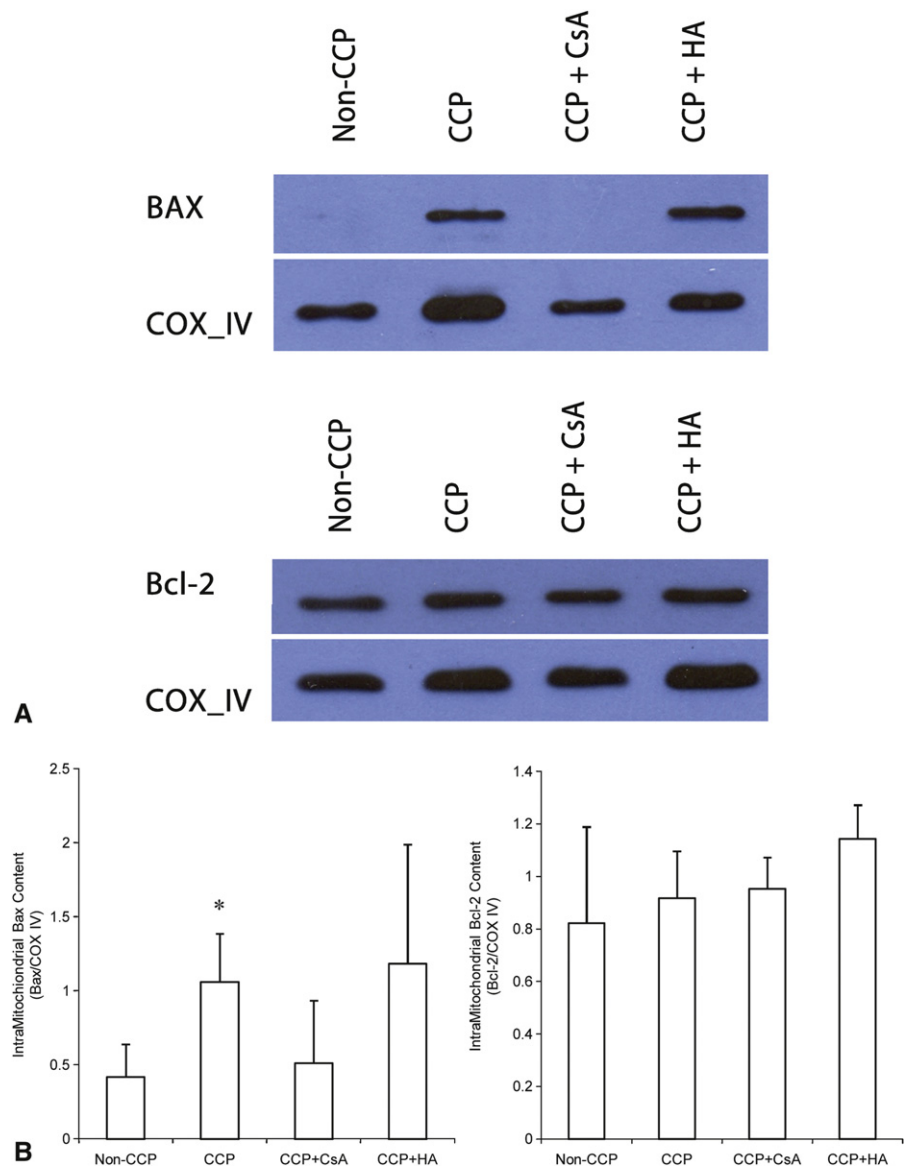
Values are presented as means ± standard deviations (n = 6 for each group). For definitions of the study groups, see the legend for Figure 1. \* $P < .01$  and † $P < .05$  versus the non-CCP group. ‡ $P < .05$  versus the CCP group. § $P = .07$  versus the CCP+HA group.

### DISCUSSION

A putative target for cardioprotection during reperfusion is the MPTP.<sup>6</sup> The MPTP remains closed throughout ischemia but opens at the time of reperfusion,<sup>20</sup> and the opening of this channel is mediated by conditions such as intracellular oxidative stress, ROS production, and calcium overload.<sup>6</sup> Sustained or irreversible opening of the MPTP leads to the dissipation of mitochondrial potential, cessation of oxidative phosphorylation, matrix swelling, and eventually outer membrane rupture.<sup>21</sup> MPTP opening can be a reversible event and does not always lead to demonstrable mitochondrial swelling<sup>22</sup> and depolarization.<sup>23</sup> Transient MPTP opening with full cardiac functional recovery is associated with mild forms of ischemia–reperfusion injury.<sup>7</sup> Inhibition of MPTP opening prevents mitochondria-driven necrotic and apoptotic processes and apoptosis-related mitochondrial dysfunction in the postcardioplegic myocardium.<sup>3</sup> The novel observations of the present study include the following: (1) transient MPTP opening occurred within 30 minutes of reperfusion after cardioplegic arrest, and MPTP resealing ensued within 30 minutes of reperfusion, and (2) precardioplegic arrest inhibition of MPTP opening by CsA prevented the occurrence of apoptosis-related mitochondrial dysfunction, whereas the facilitation of MPTP opening by the Bcl-2 inhibitor HA14-1 augmented the deficit of mitochondrial respiratory chain activity. These data suggest that MPTP opening plays an important role as a mediator of apoptosis-related mitochondrial



**FIGURE 5.** Isolated mitochondrial reactive oxygen species (ROS) production rate (RFU per minute per milligram of protein). \* $P < .05$  and \*\* $P = .06$  versus the non-CCP group. For definitions of the study groups, see the legend for Figure 1.



**FIGURE 6.** A, Representative immunoblots of mitochondrial Bax, Bcl-2, and cytochrome c oxidase IV (COX IV). \* $P < .05$  versus the non-CCP group. B, Densitometric measurements of Bax and Bcl-2 protein content normalized to COX IV. For definitions of the study groups, see the legend for Figure 1.

dysfunction after neonatal cardioplegic arrest. Although early apoptosis-related processes are initiated (Bax translocation, mitochondrial permeabilization, and cytochrome c release), we have previously demonstrated that there is sparse progression to completed apoptosis (<1% of myocytes) after 6 hours of observation in a neonatal lamb model.<sup>24</sup> Therefore the constellation of mitochondrial alterations might represent a stalled form of apoptosis, and the clinical reality that most neonatal myocardial function returns in the early postoperative period suggests that myocyte recovery (rather than cell death) is the norm.

Proposed mechanisms for mitochondrial membrane permeabilization after ischemia–reperfusion injury include nonspecific mitochondrial swelling, rupture of the outer

membrane, and MPTP opening.<sup>25</sup> The vast majority of studies evaluating these mechanisms of mitochondrial permeabilization are in infarct models of lethal mitochondrial injury with sustained MPTP opening. In contrast, cardioplegic arrest results in a mild ischemia–reperfusion injury in which recovery of myocardial function is typical and necrosis is rare or nonexistent.<sup>24</sup> However, the time course of restoration of mitochondrial integrity after cardioplegic arrest has not been fully studied. The present study suggests that resealing of the MPTP is nearly complete within 30 minutes after reperfusion and that mitochondrial electron transport is linked to the status of mitochondrial permeabilization.

Translocation of Bax to the mitochondria is a well-recognized proapoptotic signal that is associated with

mitochondrial permeabilization. Because Bax can form in vitro multimers with pore-forming capability, it has been hypothesized as a mediator of mitochondrial permeabilization in apoptotic signalling.<sup>26</sup> Our laboratory and others have demonstrated that disruption of mitochondrial membrane integrity is associated with translocation of Bax to the mitochondrial fraction.<sup>2,27</sup> In the present study we find evidence that Bax might be involved in mitochondrial permeabilization based on translocation of Bax to the mitochondrial fraction. In contrast, inhibition of MPTP opening with CsA administration is associated with mitochondrial preservation and diminished Bax translocation. These data are consistent with the concept that Bax translocation is downstream from MPTP opening and might participate in a feed-forward signal to enhance mitochondrial permeabilization.<sup>28</sup> Bax mitochondrial redistribution and cytochrome c release can occur as a delayed process in isolated cell models downstream from MPTP opening.<sup>8</sup> Bax (proapoptotic) and Bcl-2 (antiapoptotic) play opposing regulatory roles in mitochondrial permeabilization. Bax-induced mitochondrial permeability changes can be prevented by Bcl-2.<sup>29</sup> The involvement of Bcl-2 in the control of MPTP opening and apoptosis-related mitochondrial dysfunction was supported by our finding that inhibition of Bcl-2 by HA14-1 enhanced MPTP opening, and these changes were associated with deficits of activity in complexes I and IV that could be prevented by inhibition of Bax translocation with CsA.

In conclusion, our data suggest that cardioplegic arrest and reperfusion are associated with transient mitochondrial permeabilization of the MPTP and subsequent restoration of mitochondrial integrity within 30 minutes. Mitochondrial permeabilization is associated with ROS production, secondary Bax translocation, and dysfunctional electron transport at complexes I and IV, with a potential amplification role for Bax as a mediator of mitochondrial permeabilization. Therefore clinical strategies that are directed to minimize initial MPTP opening and apoptosis-related mitochondrial dysfunction might work in a synergistic fashion with other more conventional therapeutic modalities designed to minimize myocardial stunning and thereby improve postoperative myocardial performance.

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